Mechanism of polydactylly manifestation in mice and its extrapolation to humans

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ABSTRACT

The genetic polydactyly/arhinencephaly mouse (Pdn/Pdn) exhibits pre- and postaxial polydactyly and syndactyly in the extremities, absence of the olfactory bulb, agenesis of the corpus callosum and hydrocephalus. Heterozygotes (Pdn+/+) exhibit only preaxial polydactyly of distal phalangeal type in the hindlimbs and broad thumb in the forelimbs. RNase protection assay and RT-PCR analysis in Pdn/Pdn mouse embryos demonstrated reduced Gli3 gene expression. Retrotransposons were shown to be inserted into intron 3 of the Gli3 gene in the Pdn mouse. From these observations, it was concluded that the gene responsible for Pdn mouse was Gli3, mapped on mouse chromosome 13A2-3.

Human Greig cephalopolysyndactyly syndrome (GCPS) exhibits pre- and postaxial polysyndactyly and broad thumb in the extremities, broad nasal root and macrocephaly (hydrocephalus). The responsible gene has been identified as GLI3 mapped on chromosome 7p13. From the similarities in the phenotypes of GCPS and Pdn/Pdn, homology of the responsible genes (GLI3 and Gli3), and synteny regions on the chromosomes (7p13 and 13A2-3), Pdn/Pdn was suggested to be a mouse homolog of GCPS.

It is almost impossible to investigate the developmental mechanisms of human genetic congenital anomalies. The mechanisms of polydactylly manifestation investigated in the mouse homolog may be extrapolated to those involved in human disease.

Key words: Pdn mouse, limb morphogenesis, polydactyly, Gli3, Shh, GCPS

Characteristics of Pdn mouse

Polydactylly Nagoya (Pdn) was found in 1979 as a spontaneous mutant mouse. Heterozygous Pdn/+ mice show broad thumb in the forelimbs, preaxial polydactyly of distal phalangeal type in the hindlimbs and no brain abnormalities. Homozygous Pdn/Pdn mice have 2 extra digits preaxially and postaxial polydactyly in the forelimbs, and 1 extra-digit preaxially and triphalangia in the hindlimbs (Fig. 1A) (Hayasaka et al., 1980; Naruse and Kameyama, 1982). The Pdn/Pdn brain on day 0 after birth shows absence of the olfactory bulb (Fig. 1B) including absence of the corpus callosum and hydrocephalus (Naruse et al., 1990; Naruse and Keino, 1993). Polydactylly shows autosomal semi-dominant inheritance, while arhinencephaly manifestation is an autosomal recessive trait. Width of the eyes in Pdnl/Pdn mouse on day 0 is wider than that in normal neonates, indicating that Pdn/Pdn exhibits telecanthus. Pdn/Pdn neonates have cleft palate at a high frequency, and 20 % of Pdn/Pdn are exencephalic. Pdn/Pdn neonates exhibit suckling dysfunction, the cause of which was considered to be olfactory dysfunction due to non-attachment of olfactory nerve to the CNS (Naruse et al., 1994a; Hongo et al., 2000).

Responsible gene of Pdn mouse is Gli3

Responsible gene of Pdn mouse has been identified as Gli3 (Naruse and Keino, 1995). The Gli3 gene has a zinc finger motif indicating that it is a regulator gene. RNase protection assay and RT-PCR analysis exhibited the reduction of Gli3 gene expression in Pdn/Pdn embryos on day 13 (Figs. 2 and 3). Gli3 gene expression was suppressed but was not null in Pdn/Pdn mice. LA (Long-Accurate) PCR analyses indicated that at least two retrotransposons were inserted into intron 3 of the Gli3 gene. The first transposon was located 7 kb downstream of exon 3, while the last transposon was located 6 kb further downstream of the first and 3 kb upstream of exon 4 (Fig. 4). Insertion of retrotransposons into introns induces sometimes mutations by alteration of splicing, as in the case of the autoimmune LPR mouse (Kobayashi et al., 1993). In the case of the Pdn mouse, at least 5 kinds of Gli3 messenger RNA were detected (Thien and Rüther, 1999). The mouse Gli3 gene is on chromosome 13, located at 8 cM from the cen-
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The pituitary, hypothalamic hamartoma, pituitary dysplasia, and so on (Biesecker and Graham, 1996), and the responsible gene has also been shown to be GLI3 (Kang et al., 1997). Postaxial polydactyly type A, PAP-A, syndrome shows just postaxial polydactyly in both the hands and feet, and responsible gene was again reported to be GLI3 (Radhakrishna et al., 1997). Biesecker (1997) speculated that the GLI3 gene has multiple functions mediated by a zinc finger DNA binding motif (Kinzler and Vogelstein, 1990), protease cleavage site, and microtubular anchor region. The whole GLI3 protein was further speculated to function as a transcriptional activator, but after cleavage it may act as a transcriptional repressor. GCPS occurs due to mutation including the zinc finger motif, PHS is due to mutation including the protease cleavage site, and PAP-A occurs due to mutation in the microtubular anchor region (Biesecker, 1997). Human GLI3 and mouse Gli3 genes show a high degree of homology; 69% at the DNA level, and 82% at the amino acid level. On the other hand, Grzeschick's group reported a case in which a point mutation downstream of the zinc finger induced GCPS (Wild et al., 1997; Kalff et al., 1999; Radhakrishna et al., 1999). Thus, the mechanisms responsible for GCPS, PHS and PAP-A may not be as simple as originally speculated by Biesecker.

In GCPS and Pdn/Pdn, mutations are found upstream of the zinc finger motif in GLI3 and Gli3, syntenic regions of which are 7p13 and 13A2-3. Polysyndactyly, postaxial polydactyly, macrocephaly or hydrocephalus, telecanthus and frontal bossing are common phenotypes in both GCPS and Pdn/Pdn. Absence of the olfactory bulb, absence of the corpus callosum, distorted cortical plate and dysfunction of suckling behavior are observed in Pdn/Pdn, but not in GCPS. The greatest difference is that GCPS shows autosomal dominant inheritance, while Pdn/Pdn is autosomal recessive. Haplo-insufficiency of GLI3 induces GCPS in humans. However, in Pdn (Naruse and Kameyama, 1982; Naruse et al., 1990) and Xt mice (Johnson, 1967; Winter and Huson, 1988; Franz, 1994), heterozygotes exhibit mild polydactyly and no brain abnormalities, and show no phenotypic similarities to GCPS. Homozygotes exhibit similar phenotype to GCPS. Thus, there may be differences in the signal cascades downstream of the GLI3/Gli3 gene between human and mouse. Since GCPS and Pdn/Pdn have similar phenotypes, homology in their responsible genes, and syntenic regions, Pdn/Pdn is considered to be a mouse homolog of GCPS. Therefore, it may be possible to extrapolate the mechanism of polydactyly manifestation in Pdn/Pdn to that in GCPS.

**Pdn/Pdn is a homolog of GCPS**

GCPS is characterized by a peculiar facial appearance, broad nasal root and frontal bossing, macrocephaly, broad thumbs and postaxial polydactyly of the hand, preaxial polydactyly of the foot, syndactyly of the fingers and toes (Greig, 1926; Gollop and Fontes, 1985). The gene responsible for this syndrome has been identified as GLI3 (Vortkamp et al., 1991a, 1991b, 1995) Pallister-Hall syndrome, PHS, exhibits polydactyly, syndactyly, dysplastic nail, hypothalamic hamartoma, pituitary dysplasia, and so on (Biesecker and Graham, 1996), and the responsible gene has also been shown to be GLI3 (Kang et al., 1997). Postaxial polydactyly type A, PAP-A, syndrome shows just postaxial polydactyly in both the hands and feet, and responsible gene was again reported to be GLI3 (Radhakrishna et al., 1997). Biesecker (1997) speculated that the GLI3 gene has multiple functions mediated by a zinc finger DNA binding motif (Kinzler and Vogelstein, 1990), protease cleavage site, and microtubular anchor region. The whole GLI3 protein was further speculated to function as a transcriptional activator, but after cleavage it may act as a transcriptional repressor. GCPS occurs due to mutation including the zinc finger motif, PHS is due to mutation including the protease cleavage site, and PAP-A occurs due to mutation in the microtubular anchor region (Biesecker, 1997). Human GLI3 and mouse Gli3 genes show a high degree of homology; 69% at the DNA level, and 82% at the amino acid level. On the other hand, Grzeschick's group reported a case in which a point mutation downstream of the zinc finger induced GCPS (Wild et al., 1997; Kalff et al., 1999; Radhakrishna et al., 1999). Thus, the mechanisms responsible for GCPS, PHS and PAP-A may not be as simple as originally speculated by Biesecker.

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**Manifestation of polydactyly in mice**

Limb plates exhibit an apical ectodermal ridge (AER) on day 11, show mesodermal condensation of the digital anlagen on day 12.5, and show independency of the digits on day 13 of gestation in normal mouse embryos. AER formation is considered to be due to Fgf-10 from the lateral plate mesoderm (LPM) (Ouchi et al., 1997). After formation of AER, Fgf-8 from AER influences distal outgrowth of the LPM, and Fgf-10 from the LPM maintains the AER reciprocally (Celli et
Fig. 2 RNase protection assay of Gli3 gene expression in Pdn/Pdn mouse embryos on day 13 of gestation, using a riboprobe including the zinc finger domain. A 0.75 kb band of Gli3 was observed in lane 1 (+/+ embryos), but this band was very faint in lane 2 (Pdn/Pdn embryos). Control yeast RNA treated with RNase A showed no band (lane 3), and untreated yeast RNA showed a strong band of 0.93 kb riboprobe (lane 4).

Fig. 3 RT-PCR analysis of Gli3 gene expression in Pdn/Pdn embryos on day 13 of gestation. Primers were designed to amplify a fragment of 346 bp in the downstream of the zinc finger motif of the Gli3 gene. PCR product was detected in the +/+ embryos (lanes 2), but was very faint in Pdn/Pdn embryos (lanes 1). Lanes 3 shows the DNA size markers. Mouse glyceraldehyde 3 phosphate dehydrogenase (G3PDH) control amplimer set were added simultaneously as a control, and they yielded a product of 983 bp. They are enough even in lanes 1 and 2. Suppression of Gli3 gene expression was ascertained in Pdn/Pdn embryos.

Fig. 4 LA (Long-Accurate) PCR analyses indicated that at least two retrotransposons were inserted into intron 3 of the Gli3 gene in Pdn mice. The first transposon was located 7 kb downstream from exon 3, while last transposon was located further 6 kb downstream from the first, and 3 kb upstream of exon 4. Trn: transposon. E3F and E4R: primers corresponding to the sequences in the exons 3 and 4. T9R and T10F: primers corresponding to the sequences in the transposon.
al., 1998). Ohuchi et al. (1995) implanted cultured cells infected with Fgf-4-expressing retrovirus into the chick embryo LPM at the pre-limb bud stage. Extra wing formation was induced by implantation and Fgf-4 gene expression in the AER of the extra wing bud was observed. These results suggested that Fgf-4 expression in the AER induced outgrowth of the wing bud along the proximo-distal axis. At present, the role of Fgf-4 gene expression in this experiment is considered to be the substitute for the Fgf-8 gene (Ohuchi et al., 1997). After limb bud formation, Fgf-8 gene expression in the AER may induce expression of the Fgfr2c gene (receptor gene of Fgf-8) in the progress zone to cause outgrowth of the limb bud along the proximo-distal axis (Xu et al., 1998).

When zone of polarizing activity (ZPA) tissue was grafted into the preaxial region of the wing bud, mirror-image polydactyly was induced in chick embryos (Saunders and Gasseling, 1968). Thus, it has been considered that the morphogens involved in digital pattern formation along the antero-posterior axis must be secreted from the ZPA. Shh expression was observed in the ZPA region in normal mouse embryos on day 10. Therefore, Shh is a likely candidate as the morphogen from the ZPA (Riddle et al., 1993). Fgf-4 expression in the AER maintains competence of the postaxial mesodermal cells to express Shh, and Wnt-7a signal from the dorsal ectoderm probably activates Shh expression (Kawakami and Nohno, 1998). However, Shh may not be the morphogen, because it does not diffuse from the ZPA region in the limb plate. At present, Shh is considered to be the switch of the signal cascade, such as Bmp and/or Hox gene families, to form digital pattern formation along the antero-posterior axis (Francis et al., 1994). The true morphogen may be combinations of Bmp-2, -4 and -7 in the antero-posterior axis (Lyons et al., 1995). The Hox gene family including Hox-a-11, and -13 and Hox-d-11, and -13 may be the morphogen in the proximo-distal axis. Recently, Kimura et al. (2000) reported that Hox-d-13 activation by Shh is dependent on Fgf-4 and Fgf-2. Drossopoulou et al. (2000) proposed a two-step model of sequential Shh and Bmp signaling. In this model, Shh first acts on limb mesenchymal cells and primes them to be competent to form digits. Secondly, Shh induces and maintains Bmp-2 expression, which acts on the competent mesenchymal cells. Then, the Bmp family acts on the primed cells to specify digit identity. The true nature of the morphogen should be determined in the near future.

Shh expression is observed in the ZPA region in the postaxial limb bud, and Gli3 expression is observed in the limb bud except for the ZPA region in mouse embryos on day 10. These observations suggested that Gli3 and Shh gene expression down-regulate each other (Büscher et al., 1998). Recently, Wang et al. (2000) showed that Shh protein suppresses Gli3 messenger RNA and protein expression in micromass cell culture of the chick limb bud. The Pdn gene is an allele of Extra-toe, Xt gene (Schimmang et al., 1994). Xt/Xt and Xt'/Xt' show null mutation of the Gli3 gene (Schimmang et al., 1992; Vortkamp et al., 1992; Hui and Joyner, 1993), so no Gli3 gene expression was observed in the Xt/Xt limb bud on day 10 (Büscher et al., 1998).

Masuya et al. (1995) reported ectopic Shh expression preaxially in Xt/Xt'. It was speculated that ectopic Shh expression functioned as an ectopic ZPA to induce extra digits preaxially in Xt/Xt'. However, we detected neither ectopic Shh expression preaxially nor postaxial overexpression in Pdn/Pdn. Ectopic preaxial Shh expression may be too low to detect by whole mount in situ hybridization in Pdn/Pdn, since Pdn/Pdn is not a null mutation of Gli3 gene as mentioned above.

### Programmed cell death in limb morphogenesis

It has been demonstrated that programmed cell death is intimately involved in limb morphogenesis. Many zones of programmed cell death can be seen during limb morphogenesis, e.g. foyer primaire preaxial (fpp), foyer marginal I (fMI), foyer marginal V (fMV), and interdigital necrotic zone (INZ) (Milaire and Rooze, 1983). Abolishment of fpp may be the cause of preaxial polydactyly (Wise and Scott, 1982; Naruse and Kameyama, 1986a) as fpp was shown to be abolished in the genetic preaxial polydactyly mouse strains and in drug-induced preaxial polydactyly (Scott et al., 1977). Zone of programmed cell death can be observed by vital staining with Nile blue. Normal mouse embryos on day 11 showed the blue areas in the deep preaxial mesoderm of the forelimb plate (fpp), and in the preaxial AER of the hindlimb plate (Fig. 5A). However, programmed cell death in fpp and preaxial AER were abolished in Pdn/Pdn embryos on day 11 (Fig. 5B). The preaxial AER of Pdn/Pdn was thicker than that of wild-type embryos. This thickened AER was considered to be the result of delayed involution due to the altered programmed cell death in the preaxial AER. This altered programmed cell death may be the result of alteration of Bmp-4 gene expression, because Bmp-4 has been suggested to be involved in programmed cell death (Hogan, 1996). The thickened AER was suggestive of excessive Fgf-8 gene expression in the preaxial region, which may induce additional Fgfr2c or depressed Bmp-4 gene expression in the preaxial mesoderm of the progress zone. This alteration of gene expression may be sufficient to prevent the death of mesodermal cells in fpp. It has been speculated that fpp regulates the quantity of mesodermal cells in the limb plate, and therefore abolishment of fpp would result in a surplus of mesodermal cells in the preaxial region (Milaire and Rooze, 1983). However, further studies are still required to determine the detailed relationships between gene expression and programmed cell death.
Prevention of polydactyly manifestation in mouse embryos

As mentioned previously, the Shh signal switches on some signal cascades in the mesodermal cells to form each digit. In the model shown in Fig. 6, ectopic Shh expression switches on Bmp signals to cause formation of extra digits in the preaxial region using surplus mesodermal cells. Recent studies indicated that most of the mutant mouse embryos with preaxial polydactyly showed preaxial ectopic Shh expression (Masuya, 1998).

Based on these observations and speculations, we supposed that artificial cell death in the fpp region may prevent the manifestation of polydactyly in Pdn/Pdn. The fpp region in Pdn/Pdn mouse embryos was electrocauterized after demuding from the yolk sac membrane on day 11.5. After 20 hours of whole-embryo culture, the untreated left forelimb showed extra digital rays in Pdn/Pdn mouse embryos (Fig. 7A). However, the electrocauterized right forelimb showed 5 digital rays (Fig. 7B) (Naruse and Kameyama, 1986b; Naruse et al., 1994b; Naruse et al., 1997).

A similar experiment was also performed in mice using the exo utero method. After opening the uterine wall to expose the yolk sac, an electrode was inserted through the yolk sac membrane into the fpp region in the left hindlimb bud, and the fpp region was electrocauterized. After treatment, embryos inside the yolk sac were put back into the dam’s abdominal cavity. The embryos attached to the uterine wall via the placenta grew to term. Pdn/Pdn hindlimbs on day 18 after electrocauterization of the fpp region on day 11.5 were corrected to 5 digits, but still showed triphalangia (Fig. 8) (Naruse and Kameyama, 1989). These observations indicated that the fpp must be involved in determination the number of digits, but may not be concerned with the form of the thumb.

Fig. 5 Zones of programmed cell death stained with Nile blue A. A: Normal mouse embryo on day 11 of gestation. Zone of programmed cell death were observed in fpp (arrow) and preaxial AER (arrowheads). B: Pdn/Pdn embryo on day 11 showed abolition of the zones of programmed cell death in fpp (arrow) and preaxial AER (arrowheads).

Fig. 6 Model of manifestation of preaxial polydactyly. Postaxial Shh expression in ZPA region switches on Bmp signals to form 5 digits, and preaxial ectopic Shh expression switches on Bmp signals to form extra digits in the preaxial region using surplus mesodermal cells.
CONCLUSIONS

To extrapolate the mechanisms of abnormal morphogenesis in mice to human disease, it is necessary to perform investigations using an appropriate mouse homolog of human disease (Fig. 9). The human disease and mouse homolog should have similarities in their phenotypes, homology of the DNA sequences in their responsible genes, and syntenic regions in their chromosomes.

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Fig. 9  Pdn/Pdn is a mouse homolog of human GCPS. From the similarity in the phenotypes of Pdn/Pdn and GCPS, homology of the DNA sequence of the responsible genes (Gli3 and GLI3), and synteny of the chromosomes (13A2-3 and 7p13), Pdn/Pdn was suspected to be a mouse homolog of GCPS. Therefore, the mechanisms of developmental abnormalities determined in Pdn/Pdn embryos may be extrapolated to those in GCPS.

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